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ADRENERGIC-ADENOSINE 3′,5′-MONOPHOSPHATE REGULATION OF SEROTONIN N-ACETYLTRANSFERASE ACTIVITY AND THE TEMPORAL RELATIONSHIP OF SEROTONIN N-ACETYLTRANSFERASE ACTIVITY TO SYNTHESIS OF ^{3}H-N-ACETYLSEROTONIN AND ^{3}H-MELATONIN IN THE CULTURED RAT PINEAL GLAND

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ABSTRACT

KLEIN, DAVID C. AND JOAN L. WELLER: Adrenergic-adenosine 3′,5′-monophosphate regulation of serotonin N-acetyltransferase activity and the temporal relationship of serotonin N-acetyltransferase activity to synthesis of ^{3}H-N-acetylserotonin and ^{3}H-melatonin in the cultured rat pineal gland. J. Pharmacol. Exp. Ther. **186:** 516–527, 1973.

The regulation of serotonin N-acetyltransferase activity has been studied in cultured pineal glands. Addition of L-norepinephrine (NE) to cultures produces a 10- to 30-fold increase in enzyme activity after six hours of treatment. There is a plateau in enzyme activity for another six hours followed by a spontaneous decrease in enzyme activity to base-line values. NE can stimulate the enzyme over a range of 10^{-4} to 10^{-9} M concentration. Exposure to NE does not have to be constant for a long-term response. Exposure for as little as 15 minutes to NE produces about a 50% maximal response at six hours. The initial increase in N-acetyltransferase activity is blocked by cycloheximide. Cycloheximide treatment after a gland has been stimulated with NE does not cause a precipitous fall in enzyme activity but does prevent a further increase. Aliphatic amines, indoleamines, an imidazolamine and tyramine are ineffective in stimulating the enzyme. The relative potency of the compounds that stimulate enzyme activity is: L-NE $\geqq$ DL-isoproterenol > L-epinephrine > DL-octopamine > D-norepinephrine > 3,4-dihydroxyphenylamine > L-3,4-dihydroxyphenylalanine. The stimulation of enzyme activity is blocked by propranolol and is enhanced by phentolamine. This indicates that the receptor involved is a *beta* adrenergic receptor and that the response to *beta* adrenergic stimulation can be influenced by an *alpha* adrenergic mechanism. The effects of NE on N-acetyltransferase activity are mimicked by dibutyryl cyclic adenosine monophosphate (AMP), which is more effective than theophylline. Cyclic AMP is only slightly effective. The effects of dibutyryl cyclic AMP are blocked by cycloheximide but not by cyclic AMP. The stimu-

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lation of N-acetyltransferase by either NE or dibutyryl cyclic AMP is coincident in time and magnitude with the stimulation of the total production of ^{3}H-N-acetylserotonin and ^{3}H-melatonin by glands incubated with ^{3}H-tryptophan. This study describes a striking number of similarities between the factors regulating pineal adenyl cyclase, cyclic AMP, radiolabeled melatonin production from radiolabeled tryptophan, and serotonin N-acetyltransferase activity in cultured pineal glands. The findings add support to the hypothesis that melatonin production is regulated by the amount of N-acetylserotonin made available for O-methylation and that N-acetylserotonin production by serotonin N-acetyltransferase is regulated by an adrenergic-cyclic AMP mechanism.

N-acetyltransferase converts 5-hydroxytryptamine (serotonin) to N-acetyl 5-hydroxytryptamine (N-acetylserotonin), the precursor of 5-methoxy - N - acetyltryptamine (melatonin) (Weissbach *et al.*, 1960, 1961). We have found a 15- to 70-fold circadian variation in the activity of rat pineal CoA: arylamine N-acetyltransferase (E.C. 2.3.1.5) (serotonin N-acetyltransferase); high values occur at night in the dark (Klein and Weller, 1970a; Ellison *et al.*, 1972). The available evidence indicates that this increase in activity is regulated by neural signals (Klein *et al.*, 1971; Deguchi and Axelrod, 1972a). The results of our earlier organ culture studies indicated that the neurotransmitter involved in the regulation of this enzyme is norepinephrine (NE) and that it acts through an adenosine 3′,5′-monophosphate (cyclic AMP) mechanism (Klein *et al.*, 1970; Klein and Berg, 1970). In the present study we have extended and expanded these investigations of the adrenergic-cyclic AMP regulation of N-acetyltransferase activity. The temporal relationship of enzyme activity and the production of ^{3}H-N-acetylserotonin and ^{3}H-melatonin from ^{3}H-tryptophan is also described.

Experimental Procedure

Methods

Animals. Male Osborne-Mendel rats (180–220 g) were used. These animals were raised in rooms with automatically regulated lighting (light:dark 14:10), the light period starting at 5:00 A.M. The lighting intensity was 100 foot-candles. The source of light was General Electric Cool White fluorescent tubes. Pineal glands were obtained between 9:00 and 12:00 A.M. immediately after animals were stunned and decapitated.

Pineal gland organ culture. One or two pineal glands were cultured intact on the surface of a stainless-steel grid in a shallow vessel containing 0.6 ml of a modified BGJ^b medium (Klein and Weller, 1970b). Each pineal gland weighed about 1 mg. Drugs were dissolved in the medium or added in 120× concentrated 0.01 N HCl solutions. The details of organ culture of pineal glands have been presented (Klein anad Weller, 1970b). At the end of culture, pineal glands were frozen in the wells of plastic Microtest plates (Falcon Plastic Company, Los Angeles, Calif.) on Dry Ice.

Assay of N-acetyltransferase activity. Pineal glands were thawed at 4°C and homogenized individually in 30 or 40 μl of 0.1 M sodium phosphate buffer, pH 6.8, or as pairs in 50 μl of buffer. A 10-μl sample of the homogenate was added to a tube containing 10 μl of 0.1 M sodium phosphate buffer (pH 6.8), 40 nmol of acetyl CoA, and 20 nmole of ^{14}C-serotonin creatinine sulfate (specific activity, 28 c/mol). The tubes were incubated for 10 minutes at 37°C and the reaction was ended by the addition of 20 μl of an alcohol–1 N HCl (1:1) solution containing 40 nmole of each of the following: melatonin, N-acetylserotonin, hydroxytryptophol, hydroxyindoleacetic acid, methoxytryptophol and methoxyindoleacetic acid. The radiolabeled N-acetylserotonin and melatonin were isolated by thin-layer chromatography (chloroform–methanol–acetic acid, 90:10:1, in the first direction and ethyl acetate in the second direction) on precoated (0.25 mm) silica gel (F-254) thin-layer chromatography plates, 5 × 20 cm. The two areas of gel containing N-acetylserotonin and melatonin were located with short-wave ultraviolet light and removed from the plate; the associated radioactivity was counted by liquid scintillation. The assay has been described in detail (Klein, 1972). Within the conditions described here, the assay of control and NE-induced glands is linear with time and homogenate concentration (fig. 1).

Hydroxyindole-O-methyltransferase. Hydroxyindole-O-methyltransferase activity was measured by adding 10 μl of a gland homogenate (two glands per 50 μl) to a tube containing 10 μl of 0.5 M sodium phosphate buffer (pH 7.9), 0.5 nmol of N-acetylserotonin and 2 nmol of S-adenosyl-L-methionine-^{14}C-methyl (Specific activity, 55.2 c/mol) and incubating the mixture for 30 minutes. The reaction was stopped by transferring the

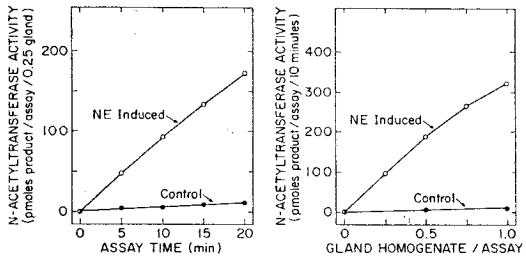


FIG. 1. Characteristics of enzyme assay. Glands were obtained from six-hour organ cultures. The concentration of NE was 10^{-4} M.

reaction solution to a 15 × 125 mm glass screw-top tube containing 2 ml of borate buffer and 10 ml of chloroform. Melatonin (O-^{14}C-methyl) was extracted into chloroform and measured (Axelrod and Weissbach, 1961).

Thin-layer chromatography. A sample of medium or gland homogenate was mixed with an equal volume of an alcohol–HCl solution containing 2 mM melatonin, 2 mM N-acetylserotonin, 2 mM hydroxytryptophol, 2 mM methoxytryptophol, 2 mM hydroxyindoleacetic acid, and 2 mM methoxyindoleacetic acid. A 20-μl sample of the mixture was applied to the corner of a 10 × 20 cm silica gel plate and chromatographed and radiolabeled products were counted as described above in the assay of N-acetyltransferase.

Data presentation. Data are presented as the mean ± standard error of the analyses of at least four pineal glands, except in figures 7 and 11. The method of data presentation for those figures is described in their legends. Student's t test was used in the statistical analysis.

Materials

Culture medium (Klein and Weller, 1970b) was purchased from Grand Island Biological Company (Grand Island, N.Y.); DL-isoproterenol·HCl, N^6,2′-O-dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) (monosodium salt) and DL-propranolol were purchased from Sigma Chemical Company (St. Louis, Mo.); cycloheximide was purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio); putrescine·2 HCl, tryptamine·HCl, cyclic AMP (free acid), tyramine·HCl, and acetyl CoA were purchased from Schwarz-Mann (Orangeburg, N.Y.); histamine (free base) was purchased from Calbiochem (Los Angeles, Calif.); ethanolamine was purchased from K & K Laboratories (Plainview, N.Y.); fraction V plasma albumin was purchased from Armour Pharmaceutical Company (Chicago, Ill.); D-norepinephrine-D-bitartrate was generously provided by Sterling-Winthrop Research Institute (Rensselaer, N.Y.), and phentolamine by Ciba Pharmaceutical Company (Summit, N.J.); DL-octopamine and all catechols and indoles not specified were purchased from Regis Chemical Company (Chicago, Ill.). The radiochemicals used and their sources were as follows: ^{14}C-serotonin creatinine sulfate (specific activity, 58 c/mol), Amersham/Searle Corporation (Chicago, Ill.); S-adenosyl-L-methionine-^{14}C-methyl) (specific activity, 56.2 c/mol), International Chemical and Nuclear Corporation (Irvine, Calif.); L-^{3}H-tryptophan (G), specific activity, 7.3 c/mmol (New England Nuclear Corporation, Boston, Mass.). The precoated chromatography plates were obtained from EM Laboratories, Inc. (Elmsford, N.Y.).

Results

Characteristics of NE stimulation of N-acetyltransferase activity. The time course of the stimulation of N-acetyltransferase by NE is seen in figure 2. After addition of NE there is a gradual increase in enzyme activity for the first six hours to a peak that is about 20-fold greater than control values. Between 6 and 12 hours of treatment, there is a plateau in enzyme activity that is followed by a gradual decrease to control values. In other experiments (*e.g.*, fig. 11), we have found that the decrease in activity cannot be prevented if NE is given every six hours. This indicates that disappearance of NE is not responsible for the decrease in enzyme

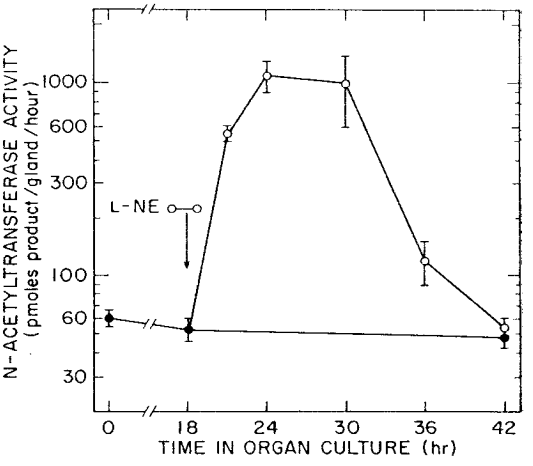


FIG. 2. Time course of NE stimulation of N-acetyltransferase activity. Glands were incubated for 24 hours under control conditions and then transferred to dishes with fresh medium. NE (10^{-4} M) was added to some of the vessel 15 minutes after the glands had been transferred. Groups of four glands were removed at the times indicated.

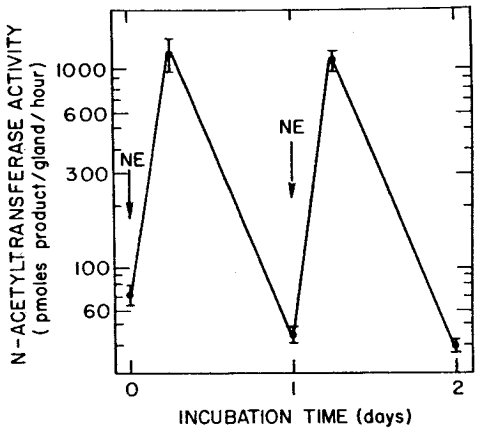


FIG. 3. Repeated stimulation of pineal glands. Glands were incubated for 0 to 48 hours. Some glands were removed at zero time, and others received NE (10^{-4} M) 15 minutes later. Groups of four glands were removed 6 and 24 hours later. The remaining glands were transferred to fresh medium at 24 hours and received NE (10^{-4} M) 15 minutes later. Groups of four glands were removed 6 and 24 hours later.

activity. The decrease also does not appear to be an indication that the cultured gland is dying because similar responses to NE (10^{-4} M) are seen when a gland receives the drug at the start of culture and again 25 hours later (fig. 3). In addition, the decrease does not appear to be due to the accumulation in the medium of a degradation product of NE or a product of an NE-stimulated gland because we have found that glands incubated in medium that had previously been incubated for 24 hours with NE and no glands, or with NE and glands, appear to respond as well to a six-hour NE (10^{-4} M) treatment as do similar glands incubated in fresh medium. Consistent with this was our finding that melatonin and N-acetylserotonin did not block NE stimulation of the enzyme. These studies certainly do not eliminate the possibility that an inhibitory substance which causes the decrease in N-acetyltransferase activity accumulates within the NE-treated pineal glands.

The dose-response relationship of NE and pineal N-acetyltransferase activity shows that this amine is effective over a wide range (10^{-4} to 10^{-9} M) of concentrations and that marked autoinhibition does not occur within this range (fig. 4). The slope of the dose-response curve presented here is typical of most experiments. However, glands occasionally respond similarly to 10^{-5} M and 10^{-7} M NE (*e.g.*, fig. 8).

The six-hour response of cultured pineal glands to NE treatment apparently is not directly proportional to the length of time NE is present in the medium (fig. 5). A 15-minute

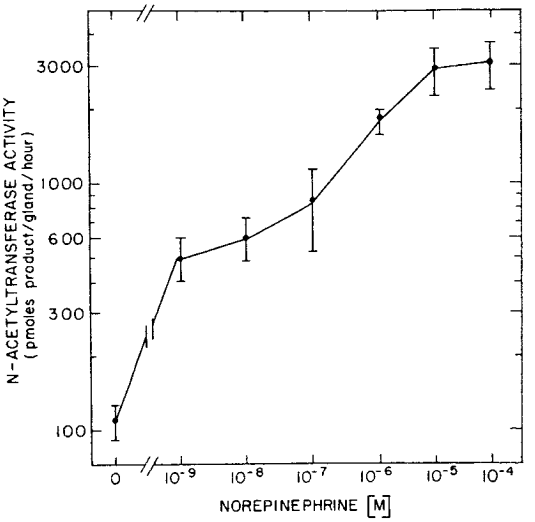


FIG. 4. Dose-response relationship of NE and N-acetyltransferase activity. Glands were cultured for 24 hours under control conditions and then transferred to vessels with fresh medium. After 15 minutes NE was added. Glands were removed six hours later.

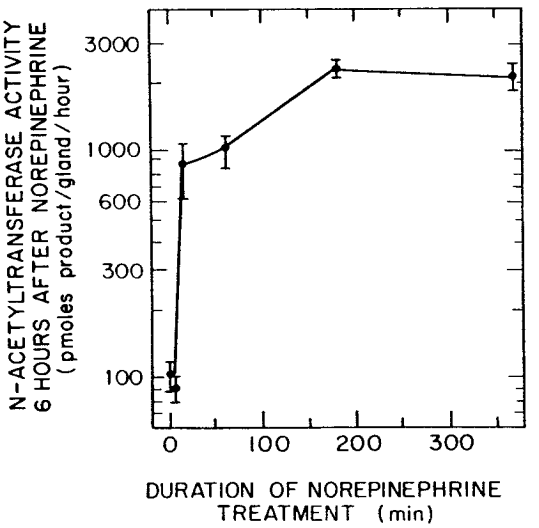


FIG. 5. Effect of duration of NE treatment on six-hour response to NE. After a 24-hour incubation period, glands were incubated for 0 to 360 minutes in medium containing NE (10^{-5} M), washed for five seconds in 25 ml of culture medium, and either frozen on Dry Ice or incubated in a vessel containing fresh medium without NE for the remainder of the 360-minute incubation period.

exposure of glands to medium containing NE (10^{-5} M) produces a response six hours later that is almost 50% of the response seen in glands treated with NE for the entire six-hour period. Apparently some event, perhaps the binding of NE to a receptor, that occurs during the first 15 minutes of culture is sufficient to direct metabolic activity for the next six hours; it appears that the continual transfer of NE from medium to glands is not necessary to sustain the increase in enzyme activity.

The stimulation of N-acetyltransferase activity by NE has been reported to be dependent upon protein synthesis (Klein *et al.*, 1970; Klein and Berg, 1970). In the present studies this was confirmed (fig. 6). It was also found that addition of cycloheximide three hours after the initial exposure of gland to NE blocks any further increase in enzyme activity but does not cause a precipitous fall in enzyme activity. It has recently been observed that there is a precipitous fall (halving time = three minutes) in enzyme activity *in vivo* at night if rats are exposed to light (Klein and Weller, 1972; Deguchi and Axelrod, 1972b). Our inability to cause a similar fall with cycloheximide, and the finding that

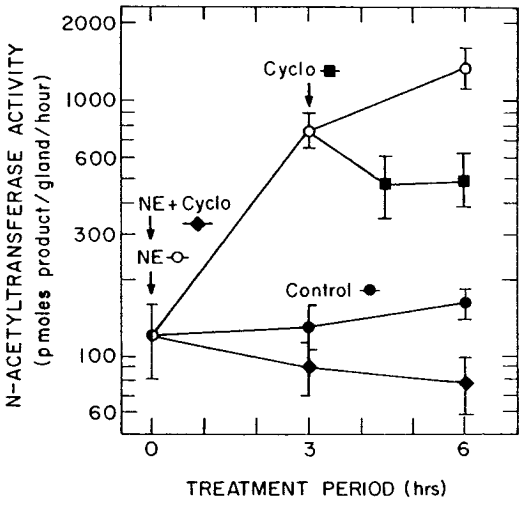


FIG. 6. Effect of inhibition of protein synthesis on N-acetyltransferase activity. Glands were incubated for 24 hours under control conditions and then transferred to media containing cycloheximide (100 μg/ml) (Cyclo) or no drug at all. After 15 minutes NE (10^{-5} M) was added to some cultures. At three hours, glands that had been treated with NE alone were washed for five seconds in 25 ml of medium and then transferred to medium containing cycloheximide.

cycloheximide does not mimic the effect of "lights-on" *in vivo* (Deguchi and Axelrod, 1972b), suggest to us that this rapid decrease observed *in vivo* is not simply the result of a cessation of all protein synthesis. The non-dramatic effect of cycloheximide after three hours of NE treatment also shows that N-acetyltransferase activity can be relatively stable inside cells in the absence of protein synthesis. The failure of N-acetyltransferase activity to decrease sharply also argues against the existence of an active, slowly turning over enzyme that rapidly degrades N-acetyltransferase.

The observations that only a 15-minute exposure to NE can direct events in the pineal gland for the next six hours, that the addition of cycloheximide just before NE treatment blocks the effects of NE, and that addition of cycloheximide after three hours of NE treatment does not produce a dramatic decrease in enzyme activity are consistent with the hypothesis that the main effect of NE is to change the steady-state levels of enzyme activity by initiating a burst of metabolic activity that increases, *via* a protein synthetic mechanism, the number of active enzyme molecules and that after a new steady state has been reached, additional NE and protein synthesis are not necessary for the persistence of the new steady state for several hours.

Specificity of the receptor involved in the stimulation of N-acetyltransferase activity. The ability of several groups of amines to stimulate the activity of N-acetyltransferase was surveyed (fig. 7). Of those compounds tested, the aliphatic amines, indoleamines, imidazolamine, and the aromatic amine, tyramine, were ineffective at 10^{-4} M. DL-Octopamine (figs. 7 and 8), the β-hydroxylated homolog of tyramine, and 3,4-dihydroxyphenylamine (dopamine) (figs. 7 and 9), the 3-hydroxylated homolog of tyramine, were as effective at 10^{-4} to 10^{-5} M as was NE. However, both DL-octopamine and dopamine were essentially ineffective at 10^{-7} M (figs. 8 and 9). L-Epinephrine was about 30% as effective as NE at 10^{-7} M (fig. 8). The D-isomer of NE was about 50% as effective as was the L-isomer at 10^{-5} M and was ineffective at 10^{-7} M (fig. 8). DL-Isoproterenol, the N-isopropyl homolog of NE, was about as effective as NE at 10^{-7} and 10^{-5} M (fig. 9). Treatment with L-3,4-dihydroxyphenylalanine (L-dopa), which

STRUCTURE-ACTIVITY RELATIONSHIPS

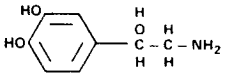
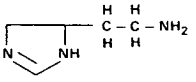
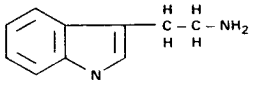
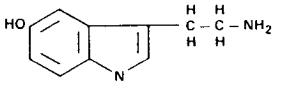
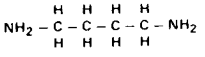
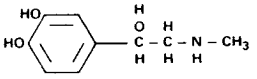
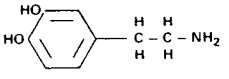
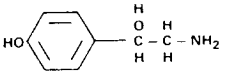
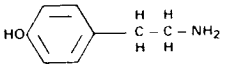
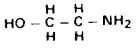
Structure	Common Name	Relative Activity of N-Acetyltransferase: Drug (0.1 mM) / Control
	L-NOREPINEPHRINE	14
	HISTAMINE	1
	TRYPTAMINE	1
	SEROTONIN	1
	PUTRESCINE	1
	L-EPINEPHRINE	19
	DOPAMINE	13
	D, L-OCTOPAMINE	9
	TYRAMINE	1
	ETHANOLAMINE	1

FIG. 7. Amine survey for stimulators of N-acetyltransferase activity. Glands were incubated for 24 hours under control conditions and then transferred to fresh medium. Drugs (10^{-4} M) were added 15 minutes later. Data are given as the treated/control ratio of the mean N-acetyltransferase activity. Each mean is based on three glands. The treatment period was three hours. There was no statistically significant difference among any of the four drug-treated groups which responded.

has recently been found to be a potent stimulator of pineal N-acetyltransferase *in vivo* (Deguchi and Axelrod, 1972b), produced only a small stimulation of enzyme activity at 10^{-4} to 10^{-5} M (fig. 9).

The receptor regulating the induction of N-acetyltransferase has the characteristics of a *beta* receptor (table 1). The NE stimulation of enzyme activity was blocked by propanolol, a known *beta* blocker, but was not blocked by phentolamine, a known *alpha* blocker. Phentolamine had the opposite effect; pretreatment of glands with it enhanced ($P > .05$) the response of glands to NE. These findings are consistent with the *in vivo* observations of Deguchi and Axelrod (1972b). They have also found that a

single injection of propranolol mimicks the effect of light at night, suggesting that displacement of NE from a receptor is necessary of the light-induced rapid increase in N-acetyltransferase activity, and that continual NE stimulation, *i.e.*, occupation of a receptor, is necessary to maintain induced N-acetyltransferase levels in the intact gland. However, in unpublished studies we have not been able to reverse the effects of a six-hour treatment with NE in organ culture with propranolol. This leads us to suspect that another event in addition to the removal of NE from a receptor must be necessary for the rapid decrease in enzyme activity.

Effects of cyclic AMP and related compounds on the activity of N-acetyltransferase. The relative effects of treatment of cultured pineal glands with cyclic AMP, dibutyryl

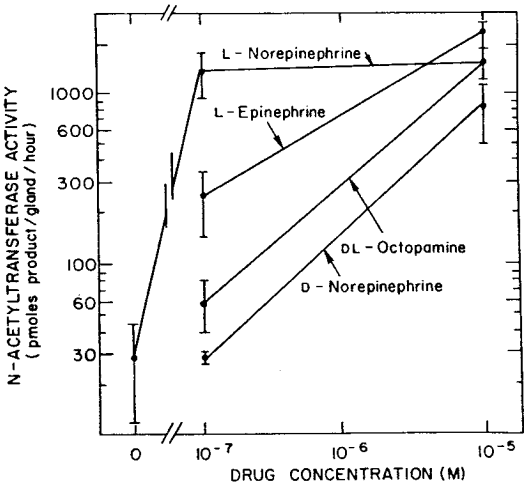


FIG. 8. Dose-response relationship of drug and N-acetylytransferase activity. Glands were incubated for 24 hours under control conditions and then transferred to vessels with fresh medium. Drugs were added 15 minutes later. The treatment period was six hours.

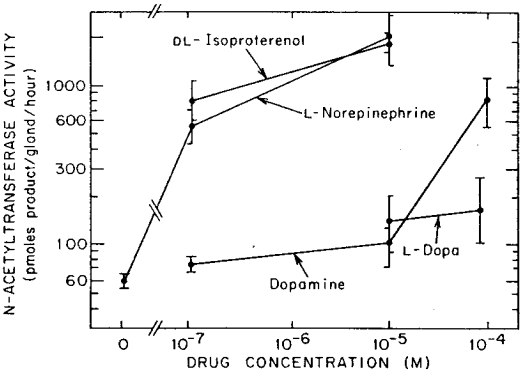


FIG. 9. Dose-response relationship of drugs and N-acetylytransferase activity. Glands were incubated for 24 hours under control conditions and then transferred to vessels with fresh medium. The drugs were added 15 minutes later. The treatment period was six hours.

TABLE 1

Effect of adrenergic blocking agents on N-acetyltransferase activity in cultured rat pineal glands

Pineal glands were cultured under control conditions for 24 hours and then transferred to vessels with fresh medium for a 6.75-hour treatment period. Some of the medium contained propranolol or phentolamine. NE was added 0.75 hours later.

Experiment	Treatment		Response
	24.00–24.75 hr	24.75–30.75 hr	
			pmol/gland/hr
1	Control	Control	111 ± 54
	Control	Norepinephrine (10^{-6} M)	1054 ± 244
	Propranolol (10^{-4} M)	Propranolol (10^{-4} M) + norepinephrine (10^{-6} M)	88 ± 21
	Phentolamine (10^{-4} M)	Phentolamine (10^{-4} M) + norepinephrine (10^{-6} M)	1955 ± 266[a]
2	Control	Control	164 ± 21
	Control	Norepinephrine (10^{-6} M)	422 ± 45
	Phenotlamine (10^{-4} M)	Phentolamine (10^{-4} M)	111 ± 20
	Phentolamine (10^{-4} M)	Phentolamine (10^{-4} M) + norepinephrine (10^{-6} M)	1092 ± 200[a]

[a] Value for phentolamine + NE is greater than that for NE, $P > .05$.

cyclic AMP and theophylline are seen in figure 10. Dibutyryl cyclic AMP, an inhibitor of pineal phosphodiesterase (Klein anad Berg, 1970), is the most effective of this group. It produced a response at 10^{-3} M that was similar to that produced by NE at 10^{-5} M (fig. 10a). Lower concentrations had little effect. Theophylline had no effect at 10^{-3} M but did stimulate N-acetyltransferase activity at 10^{-2} M (fig. 10b). Cyclic AMP at this concentration had only a slight stimulatory effect on N-acetyltransferase activity (fig. 10). In this series of studies, cycloheximide treatment also completely blocked the effects of a six hour treatment with dibutyryl cyclic AMP (unpublished studies) which is consistent with earlier reports (Klein and Berg, 1970). The stimulation of N-acetyltransferase by dibutyryl cyclic AMP was not inhibited by 10^{-3} M cyclic AMP (fig. 10b).

The temporal relationship of NE- and dibutyryl cyclic AMP-stimulated changes in serotonin N-acetyltransferase activity and the concentration of ^{3}H-N-acetylserotonin and ^{3}H-melatonin in the gland and medium. The alterations in the activity of N-acetyltransferase due to treatment with either NE or dibutyryl cyclic AMP were observed to be the same in this study (fig. 11). The 10- to 15-fold increase in enzyme activity was accompanied by sharp increases in the gland content of ^{3}H-N-acetylserotonin and ^{3}H-melatonin that were about the same magnitude. There was an equally rapid increase in the amount of these compounds in the culture medium three to nine hours after the treatment with drugs was initiated. Earlier effects of drugs may not have been detected because of the large dilution by the culture medium. In contrast to the large effects detailed above, there was only a 1.3- to 1.4-fold increase in the activity of hydroxyindole-O-methyltransferase.

Comparison of the gland content of ^{3}H-N-acetylserotonin or ^{3}H-melatonin, which is no greater than 30 pmol, and the amount released into the medium, which is about 600 pmol, indicates that there must be a very rapid turnover of these compounds in the gland in organ culture. It also appears that production regulates storage and release of both of these compounds *in vitro* because storage and release are seen to decrease sharply when production stops.

It was observed in this study that dibutyryl cyclic AMP treatment, as is true of NE treatment, results in first an increase in enzyme activity, then a plateau, and then a decrease. This indicates that the mechanism involved in this autoregulated decrease appears to be associated with a process common to both NE and dibutyryl cyclic AMP stimulation of N-acetyltransferase activity.

Discussion

Adrenergic regulation of N-actyltransferase. The observation that the treatment of a cultured pineal gland with NE causes a stimulation of N-acetyltransferase, an example of neural regulation of gene expression, is of special interest and importance because of the evidence, in part presented in this report, which indicates 1) that this is a reproduction of a physiological event which occurs daily in the rat and 2) that this enzyme has an important role in the regulation of indole metabolism in the pineal gland. This evidence will be discussed below. The ques-

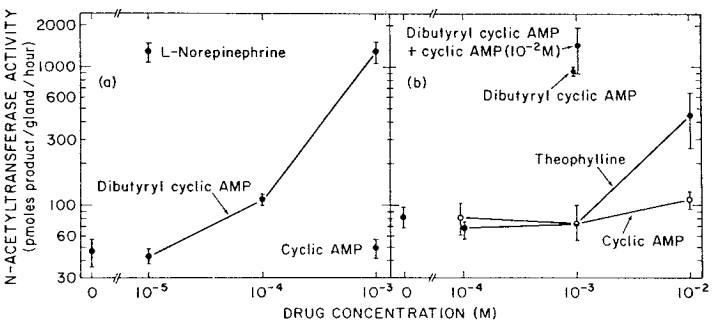


FIG. 10. Dose-response relationship of drugs and N-acetyltransferase activity. Glands were incubated for 24 hours under control conditions and then transferred to vessels with fresh medium containing the indicated drug or no drug; NE was added 15 minutes later. The treatment period was six hours.

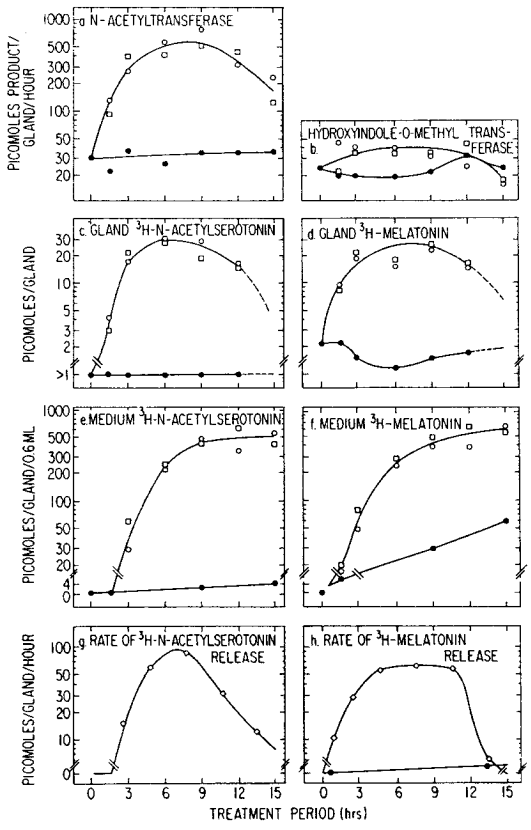


FIG. 11. Time course of the effects of NE and dibutyryl cyclic AMP treatment on (a) N-acetyltransferase activity, (b) hydroxyindole-O-methyltransferase activity, (c) gland ^{3}H-acetylserotonin, (d) gland ^{3}H-melatonin, (e) medium ^{3}H-N-acetylserotonin, (f) medium ^{3}H-melatonin, (g) rate of ^{3}H-N-acetylserotonin release, and (h) rate of ^{3}H-melatonin release. Glands were incubated for 24 hours in medium containing L-^{3}H-tryptophan (G) (10 μc/ml, 50 μc/μmol) and then were transferred to vessels containing fresh medium with L-^{3}H-tryptophan. There were two glands/vessel. Some media contained dibutyryl cyclic AMP (10^{-3} M) (○——○), some media received NE (10^{-4}) 15 minutes later (□——□), and the remaining vessels were controls (●——●). Each datum point in parts a to f is the average of two determinations performed on 10-μl samples of either a 50-μl homogenate of two glands or the medium from duplicate cultures. Each datum point in parts g and h is calculated from the data in parts e and f, respectively. The symbols (◇——◇) in parts g and h represent the pooled NE and dibutyryl cyclic AMP data in parts e and f. The release of ^{3}H-N-acetylserotonin by control glands was essentially undetectable and was disregarded in the calculation of release.

tion of physiological importance of this mechanism has been discussed elsewhere (Klein, 1973).

Under normal conditions the activity of serotonin N-acetyltransferase in an intact pineal gland increases in darkness at night (Klein and Weller, 1970a). Apparently this is initiated by a neural signal from a central nervous system nucleus which stimulates nerve cell bodies in the superior cervical ganglia (Klein *et al.*, 1971). This appears to result in the transmission of a signal *via* postganglionic nerve fibers which causes the release of a transmitter from the sympathetic nerve fiber network pervading the pineal gland. The evidence supporting this is that removal of the superior cervical ganglia or removal of input to the ganglia blocks the increase usually observed at night (Klein *et al.*, 1971). Evidence that NE is the physiological transmitter of this effect is found in the present study, which has shown that the receptor involved in this response is far more sensitive to L-NE than to other amines, such as serotonin, histamine, dopamine and octopamine, that are contained in the pineal gland or other sympathetically innervated structures (Giarman and Day, 1958; Pelligrino de Iraldi and Zieher, 1966a; Molinoff and Axelrod, 1969; Prop and Ariens-Kappers, 1961). In addition, NE is known to be located in the pineal sympathetic nerve network (Wolfe *et al.*, 1962; Bondareff and Gordon, 1966; Pelligrino de Iraldi and Zieher, 1966b). The apparent sensitivity of the NE-receptor regulating N-acetyltransferase activity indicates that only a small fraction (less than 5%) of the 11 pmol of NE normally contained in a functioning pineal gland at 7:00 P.M. (Moore *et al.*, 1968) would have to be released into the extracellular space to cause a near-maximal (equivalent to 10^{-6} M NE treatment in culture) stimulation of N-acetyltransferase activity. This statement is based on the assumptions that the volume of a pineal gland is about 1 μl and the extracellular space is 10 to 30% of this. This dose-response relationship is consistent with a physiological role of NE as the neurotransmitter regulating N-acetyltransferase activity.

Mediation by cyclic AMP. The mechanisms through which NE stimulates N-acetyltransferase activity clearly involve adenylate cyclase-cyclic AMP mediation. This conclusion is based partly on a comparison of the specificity of the receptor regulating pineal adenylate cyclase and N-acetyltransferase activity (Weiss and Costa, 1968). Both enzymes are stimulated by L-NE

better than by D-NE or dopamine, and are also stimulated by L-epinephrine and DL-isoproterenol but not by histamine or serotonin. The total amount of cyclic AMP in a cultured pineal gland can be also increased by treatment with NE; this effect, like the NE stimulation of adenylate cyclase and N-acetyltransferase, is blocked by a *beta* adrenergic blocker but not by an *alpha* adrenergic blocker (Strada *et al.*, 1972). The apparent enhancement of NE stimulation of N-acetyltransferase activity by the *alpha* adrenergic blocker may not involve cyclic AMP. In contrast to some tissues (Turtel and Kipnis, 1967), this class of blocking agents has not been observed to enhance NE stimulation of either adenylate cyclase in pineal homogenates or cyclic AMP in cultured pineal glands (Weiss and Costa, 1967; Strada *et al.*, 1972). However, the studies on the effect of an interaction of adrenergic blocking agents and NE on adenylate cyclase and cyclic AMP in the pineal gland have not been extensive. A more detailed examination of this relationship might uncover subtle effects of *alpha* adrenergic blocking agents. The opposing roles of *alpha* and *beta* adrenergic receptors in cyclic AMP-mediated events has been recently reviewed and seems to be a common situation (Robison *et al.*, 1971). However, the mechanism underlying this, especially in the pineal gland, remains unclear.

Further evidence that cyclic AMP is involved in the adrenergic stimulation of N-acetyltransferase comes from the studies with cyclic AMP and related compounds. Both dibutyryl cyclic AMP and theophylline, inhibitors of pineal cyclic nucleotide phosphodiesterase (Klein and Berg, 1970), can stimulate N-acetyltransferase activity. Theophylline is a less effective stimulator than dibutyryl cyclic AMP. This may be related to the observation that theophylline is also a less effective inhibitor of pineal cyclic nucleotide phosphodiesterase than is dibutyryl cyclic AMP (Klein and Berg, 1970). The failure of cyclic AMP to stimulate N-acetyltransferase may be explained by the evidence that cyclic AMP is rapidly metabolized by pineal glands in culture. Most of the radioactivity in a gland cultured with ^{3}H-cyclic AMP is recovered as ^{3}H-cyclic AMP derivatives (Berg and Klein, 1971). An additional explanation for the lack of an effect of cyclic AMP is that an inhibitor of the effects of cyclic AMP may be produced that is a derivative of cyclic AMP. Evidence in support of this taking place in other tissues has been presented by Murad (Murad *et al.*, 1969). However, in the present study we found that addition of 10^{-2} M cyclic AMP to medium also containing dibutyryl cyclic AMP did not reduce the effectiveness of the latter compound at the time examined. This argues against the possibility of autoinhibition (*i.e.*, that cyclic AMP did not act because in its presence an inhibitor of the effects of cyclic AMP was formed which blocked stimulation of N-acetyltransferase).

Another finding in support of the cyclic AMP mediation of the effects of NE on N-acetyltransferase activity is that dibutyryl cyclic AMP mimics the effects of NE (Klein *et al.*, 1970; Berg and Klein, 1971). The time course of stimulation by both compounds is essentially identical, and the stimulatory effects of either compound are blocked by cycloheximide.

Role of N-acetyltransferase in indole metabolism. As noted earlier, N-acetyltransferase converts serotonin to N-acetylserotonin, which is the precursor of melatonin. It appears from *in vivo* and *in vitro* studies that large changes in the activity of this enzyme control major changes in indole metabolism (Klein and Weller, 1970a). For example, when N-acetyltransferase activity is 15 to 70-fold higher at night, the amount of serotonin in a pineal gland is about 50% of the day values (Klein and Weller, 1970a) and the amounts of N-acetylserotonin (Klein and Weller, 1972) and melatonin (Reiter *et al.*, 1971) are at least 10-fold greater than day values.

Melatonin synthesis has been studied in organ culture by measuring the amount of ^{14}C-melatonin that is formed by ^{14}C-tryptophan (Axelrod *et al.*, 1969). Striking similarities between the receptor regulating ^{14}C-melatonin synthesis and N-acetyltransferase exist. Both parameters are stimulated by L-NE (Axelrod *et al.*, 1969) and this stimulation is blocked by a *beta* adrenergic blocker (Wurtman *et al.*, 1971); histamine, tryptamine and serotonin are ineffective (Axelrod *et al.*, 1969). The NE stimulation of either parameter is blocked by cycloheximide (Klein *et al.*, 1970). However, there are some inconsistencies in the comparison of NE stimulation of N-acetyltransferase and of ^{14}C-melatonin production. In the present report it was seen that D-norepinephrine, DL-octopamine and

dopamine are relatively poor stimulators of N-acetyltransferase activity compared with NE; tyramine was without effect. However, it was previously found that all of these compounds are about as good as or better than NE is as stimulators of ^{14}C-melatonin synthesis (Axelrod *et al.*, 1969). The explanation of these differences may reside partly in the difference in the organ culture techniques used by the laboratories resulting in different metabolic rates of degradation or conversion of the amines. However, in the case of tyramine, and perhaps the other compounds, a more probable explanation involves interaction with stored NE. Tyramine is known to very effectively displace NE from storage sites in nerve endings (Muscholl, 1966). In the studies in which the specificity of compounds stimulating ^{14}C-melatonin synthesis was studied, freshly removed pineal glands were added to culture medium containing drugs (Axelrod *et al.*, 1969). At this time tyramine could rapidly act *via* the NE displacement mechanism. In the studies presented here, drugs were added after glands had been incubated for 24 hours. During this time it is highly likely that the nerve endings would have degenerated because they were deprived of their cell bodies. Under these conditions, there would be no stored NE available for displacement by tyramine or any other amine.

The *in vitro* demonstration that dibutyryl cyclic AMP, as was true of NE, could stimulate both N-acetyltransferase activity and radiolabeled melatonin synthesis from radiolabeled tryptophan provides a basis for the proposal that NE physiologically regulates melatonin *in vivo* synthesis by regulating N-acetyltransferase activity *via* a cyclic AMP mechanism (Shein and Wurtman, 1969; Klein *et al.*, 1970). Additional support for this can be seen in the almost identical dose-response relationships of dibutyryl cyclic AMP with either N-acetyltransferase activity or ^{3}H-melatonin production (Berg and Klein, 1971); both processes are also stimulated by theophylline. Comparison of the time courses of the NE and dibutyryl cyclic AMP stimulation of N-acetyltransferase activity, gland content of ^{3}H-melatonin and release into the medium of ^{3}H-melatonin indicates a remarkable similarity in the time course and magnitude of these responses. In addition, it was seen that ^{3}H-N-acetylserotonin in the gland and the medium rose and fell coincidentally with the activity of N-acetyltransferase and ^{3}H-melatonin synthesis. This is consistent with the hypothesis that melatonin synthesis is regulated by the amount of N-acetylserotonin available for O-methylation by hydroxyindole-O-methyltransferase (HIOMT) (Klein *et al.*, 1970). The activity of the latter enzyme was seen to increase following treatment of cultured pineal glands with dibutyryl cyclic AMP or NE. Small effects of NE on this enzyme have been seen before (Klein and Berg, 1970). However, previous published (Berg and Klein, 1971) and unpublished studies have not detected any significant change in the activity of this enzyme due to dibutyryl cyclic AMP which leaves the cyclic AMP regulation of HIOMT in question. The small increase in HIOMT activity appears insignificant relative to the many-fold increase in N-acetyltransferase activity in a consideration of the enzymatic regulation of large changes in ^{3}H-melatonin production. It would appear that HIOMT could limit the maximum amount of melatonin synthesized, but that it is not responsible for causing large changes in melatonin production which are apparently regulated by changes in the production and concentration of N-acetylserotonin.

The mechanism through which cyclic AMP regulates N-acetyltransferase is not clear. It seems to involve new protein synthesis. Perhaps cyclic AMP acts to stimulate the net production of N-acetyltransferase molecules, to increase formation of an N-acetyltransferase activating enzyme, or inhibit a degrading enzyme. It is also unclear why the activity of N-acetyltransferase falls after 12 hours of stimulation with either NE or dibutyryl cyclic AMP. One possibility is that an endogenous inhibitor is gradually formed. Another possibility is that an N-acetyltransferase-stabilizing substance is gradually consumed, and when the concentration of it drops below a critical level, N-acetyltransferase activity drops.

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